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Remarks

The December 16, 2003 Official Action has been carefully reviewed. In view of the amendments submitted herewith and the following remarks, favorable reconsideration and allowance of this application are respectfully requested.

At the outset it is noted that a shortened statutory response period of three (3) months was set forth in the December 16, 2003 Official Action. Therefore, the initial due date for response was March 16, 2003. A petition for a 3 month extension of the response period is presented with this response, which is being filed before the expiration of the three month extension period.

At page 3 of the Official Action, the Examiner has objected to the specification for allegedly failing to describe both Figures 3A and 3B in the Brief Description of the Drawings and for allegedly not identifying the significance of the arrow in Figure 4B. Applicants have addressed the Examiner's objections by amending the description of Figures 3 and 4. Support for the amendment to the description of Figure 3 can be found, for example, within Figures 3A and 3B. Support for the amendment to the description of Figure 4 can be found at page 17, lines 9-15.

The Examiner has also objected to claims 43, 46, and 54 in view of certain typographical errors. Applicants have cancelled claims 43 and 46 and have amended claim 54 to replace the term, "molecules," with "modules," as suggested by the Examiner.

At pages 5-9 of the Official Action, the Examiner has rejected claims 20, 23-25, 29, 30, and 43-63 under 35 U.S.C. §112, second paragraph for alleged indefiniteness on various grounds.

The Examiner has also rejected claims 20, 23-25, 29, 30, and 43-63 for allegedly failing to satisfy the written description requirements under 35 U.S.C. §112, first paragraph

on various grounds.

Claims 46, 48, 52, and 53 have been rejected under 35 U.S.C. §102(b) as allegedly anticipated by Kuhstoss et al. (Gene (1996) 183:231-236).

Lastly, the Examiner has rejected claims 20, 23-25, 29, 30, 43-45, 47 and 49 under 35 U.S.C. §103 as allegedly unpatentable over U.S. Patent 5,712,146 in view of Khosla et al. (Chemical Reviews (1997) 97:2577-2590).

The foregoing rejections constitute all of the grounds set forth in the December 16, 2003 Official Action for refusing the present application.

In accordance with this amendment, claims 20, 23-25, 29, 30, and 43-53 have been cancelled. All of the rejections outstanding with respect to these claims are, therefore, rendered moot. The cancellation of claims 20, 23-25, 29, 30, and 43-53 should not be construed as indicative of Applicants' concurrence or acquiescence in the various rejections of claims 20, 23-25, 29, 30, and 43-53 as set forth in the December 16, 2003 Official Action, or otherwise as an abandonment of Applicants' efforts to secure patent protection on the subject matter of claims 20, 23-25, 29, 30, and 43-53. To the contrary, Applicants vigorously dispute those grounds of rejection. Such arguments as Applicants have to advance in rebuttal, however, are being reserved for a continuing application, which is expected to be filed and include claims directed to the subject matter of cancelled claims 20, 23-25, 29, 30, and 43-53.

Claims 54, 60, and 63 have been amended in accordance with this amendment. The amendments to claim 54 are intended to make the claim more closely approximate the proposed draft claim provided by the Examiner. Claims 60 and 63 have been amended to expressly recite the acyltransferase domains specific for loading ethylmalonyl, allyl and hydroxymethyl units. Support for these amendments can be

found, for example, at page 27, lines 7-14 of the present specification.

New claims 64-76 are included with this amendment. These new claims are directed to polyketide synthases comprising specifically identified loading modules or loading module domains. Support for newly added claims 64-76 can be found throughout the specification. Specifically, support for claims 64-70 and 72 can be found at original claim 46; page 27, lines 7-14; and page 47, lines 7-10. Support for claim 71 can be found, for example, at page 21, lines 1-9 and original claim 13. Support for claims 73, 74, and 76 can be found, for example, in original claims 7 and 46. Support for claim 75 can be found, for example, at claim 43 and page 22, lines 17-22.

No new matter has been introduced into this application by reason of any of the amendments presented herewith.

CLAIMS 20, 23-25, 29, 30, AND 43-63, AS AMENDED, MEET THE REQUIREMENTS UNDER 35 U.S.C. §112, SECOND PARAGRAPH

The Examiner has rejected claims 20, 23-25, 29, 30, and 43-63 under 35 U.S.C. §112, second paragraph for alleged indefiniteness on the following grounds.

First, it is the Examiner's position that independent claims 43, 46, and 54 are narrative and indefinite. Applicants have adopted the helpful suggestions set forth in the Examiner's proposed draft claim in amending claim 54. Notably, Applicants have cancelled claims 43 and 46 and have written new claims 64-76 with the Examiner's suggestions in mind.

Second, it is the Examiner's position that the term "residue" in independent claims 43, 46, and 54 is confusing when used to refer to anything other than an amino acid residue. Applicants respectfully disagree with the Examiner

and submit that a skilled artisan would readily recognize the meaning of the term "residue" as it was used throughout the claims. However, in the interest of expediting prosecution, Applicants have adopted the Examiner's suggestion and amended all claims to recite a "moiety" in place of "residue" when the entity referred to is not an amino acid residue.

Third, the Examiner contends that, in claims 43, 46, and 54, the phrase "wherein at least the first of said extension modules is not naturally associated with a loading module that effects decarboxylation of an optionally substituted malonyl" is unclear. Applicants respectfully take exception to the Examiner's contention that "virtually all loading modules in nature ... effect decarboxylation of an optionally substituted malonyl." Indeed, as of the priority date of the present invention, skilled artisans were not aware that loading modules that did contain a KSq domain had the recited functionality. However, in the interest of advancing the present prosecution, Applicants have amended claim 54 to recite "said loading module" in place of "a loading module," as suggested by the Examiner.

The Examiner also alleges that it is unclear what part of the loading domain needs to be "not naturally associated" with at least the first extension module. Applicants respectfully disagree with the Examiner's interpretation of the claims in question, which, as amended, recite the phrase "not naturally associated with said loading module." Literally interpreted, the claims clearly do not refer to extension modules not naturally associated with **part** of the loading module, but rather the loading module as a whole. Accordingly, Applicants submit that the claims, as amended, plainly refer to the loading module as a unit and should not be naturally associated with at least the first of the extension modules. Such an interpretation is also consistent with the examples identified by the Examiner at

page 7 of the instant Official Action. Applicants also maintain that a skilled artisan would be readily able to determine whether or not an extension module is naturally associated with a loading module.

Fourth, the Examiner asserts that the metes and bounds of the term "corresponding to" are unclear. Applicants have deleted the allegedly objectionable term from the claims and thereby obviated this rejection.

Fifth, it is the Examiner's position that the term "a cysteine in the active site" is indefinite because the term suggests that there is more than one cysteine in the active site. Here again, in the interest of advancing the prosecution of the application, Applicants have employed the Examiner's suggestion and replaced the allegedly indefinite term with "the cysteine residue in the active site."

Sixth, the Examiner has rejected claims 20, 23-25, 55, and 56 for allegedly lacking proper antecedent basis for the term "the acyltransferase domain." Applicants have amended the claims to recite "said acyltranferase domain," so as to more clearly refer to the acyltransferase domain of the loading domain and not of an extension module.

Lastly, the Examiner has rejected claims 46-53 and claims 50 and 51 for recitation of the allegedly indefinite terms "derived from" and "corresponds to," respectively. Applicants have cancelled claims 46-53 thereby obviating these rejections.

In light of all of the foregoing, Applicants submit that the rejection of claims 20, 23-25, 29, 30, and 43-63 under 35 U.S.C. §112, second paragraph for alleged indefiniteness is untenable and request its withdrawal.

CLAIMS 20, 23-25, 29, 30, AND 43-63, AS AMENDED, SATISFY THE WRITTEN DESCRIPTION REQUIREMENT UNDER 35 U.S.C. §112, FIRST PARAGRAPH

The Examiner has rejected claims 20, 23-25, 29, 30, and 43-63 for allegedly failing to satisfy the written description requirements under 35 U.S.C. §112, first paragraph on the following grounds.

The relevant inquiry in determining compliance with the written description requirement of 35 U.S.C. §112, first paragraph, is whether the originally filed specification reasonably conveys to a person having ordinary skill in the art that applicant had possession of the claimed subject matter. In re Kaslow, 217 U.S.P.Q. 1089 (Fed. Cir. 1983).

Furthermore, the Examiner has the initial burden of presenting evidence or reasons why a person of skill in the art would not recognize in applicants' specification disclosure a description of the invention defined by the claims. Ex parte Sorenson, 3U.S.P.Q.2d 1462 (BD. Pat. App. 1987).

In the instant Official Action, the Examiner contends that the recitation of using **any** acyltransferase domain that is specific for loading ethylmalonyl, as in claims 25, 49 and 60, is not supported by the specification. Applicants respectfully disagree with the Examiner's position, inasmuch as the recitation of the specific examples of the acyltransferase domains of extension modules 5 of both the spiramycin and monensin polyketide synthases (see page 27, lines 12-14) is adequate to describe the domains in sufficient detail for a skilled artisan to conclude that Applicants had possession of the claimed subject matter. However, in the interest of expediting prosecution of the instant application, Applicants have amended claim 60 to recite the specific examples set forth at page 27, lines 12-14. Claims 25 and 49

have been cancelled.

Second, the Examiner has rejected claims 30, 53, and 63 for allegedly failing to comply with the written description requirement under 35 U.S.C. §112, first paragraph. The Examiner contends the specification does not provide support for any polyketide synthase having a side chain of an allyl or hydroxymethyl group. Applicants again take issue with the Examiner's position, given that the recitation of the specific examples at page 27, lines 7-12 provides a skilled artisan with sufficient information to conclude that Applicants had possession of the claimed subject matter. However, Applicants have amended claim 63 to recite the specific examples set forth at page 27, lines 7-12. Notably, claims 30 and 53 have been cancelled.

Third, it is the Examiner's position that the specification fails to provide any specific hybrid polyketide synthases that produce 12-member macrolides. The Examiner argues that "no mention of naturally-occurring PKSs that produce 12-membered macrolides is found" in the specification. Applicants strenuously disagree with the Examiner in this regard. Indeed, throughout the specification, methymycin is provided as a "particularly suitable PKS" of the instant invention (see, for example, page 20, lines 14-24; page 21, lines 10-15; and page 21, line 23 through page 22, line 3) and is well-known in the art to be a 12-membered macrolide (see, for example, abstract of Cane et al. (1993) J. Am. Chem. Soc. 115:522-526 (copy attached)). The present specification plainly describes not only a representative species of the claimed genus, but also provides more than adequate disclosure of the physical and functional characteristics of the polyketide synthases. Thus, Applicants respectfully submit that the rejection of claims 20, 23-25, 29, 30, 43, 44, 53, and 63 for alleged lack of written description is untenable.

Fourth, the Examiner has rejected claims 20, 23-25,

30, and 43-63 because the specification allegedly does not provide support for an AT domain which is "adapted to load" the starter unit onto the ketosynthase domain. The Examiner contends that the specification fails to identify particular characteristics of AT domains "adapted" to KSq domains. In this response, Applicants have amended the claims to replace the phrase "adapted to load an optionally substituted malonyl" with "loads an optionally substituted malonyl." As mentioned in the specification, any "AT domain derived from any extension module of a Type I PKS" can be employed so long as there is matching specificity with the KSq domain in terms of the loading units (see page 23, lines 2-7). Furthermore, Examples 11-15 demonstrate the successful production of a polyketide from a polyketide synthase comprising a KSq domain from the oleandomycin polyketide synthase fused to an AT domain of the rapamycin polyketide synthase. Thus, Applicants submit that the specification provides adequate guidance for the selection and combination of AT and KS domains that are not naturally associated and conveys to a skilled artisan that Applicants were in possession of the claimed subject matter as of the filing date of this application. Accordingly, Applicants respectfully request the rejection of claims 20, 23-25, 30, and 43-63 be withdrawn.

Lastly, the Examiner has rejected claims 54-63 because the specification allegedly fails to teach hybrid polyketide synthases comprising engineered KSq domains. Applicants vigorously dispute the Examiner's position in this regard.

As noted above, the written description requirement under 35 U.S.C. §112, first paragraph, is satisfied when the claimed subject matter is described in the specification in such a way as to convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. There is no

requirement for a claim to a genus that a representative number of species has actually been disclosed or actually reduced to practice, provided there is sufficient information in the application for a person of skill in the art to understand or appreciate that the inventors were in possession of the claimed invention and that a correlation between the function and structure of the claimed genus is shown.

In the present application, Applicants have clearly described the functional characteristics of the claimed invention, including the decarboxylative activity of the KSq domain, coupled with a disclosed correlation between structure and function, such as the alteration of the cysteine residue to a glutamine residue results in said activity (see, for example, page 18, lines 12-20; page 16, line 22 through page 17, line 15; and Figure 4). Further, explicit instructions for generating an engineered KSq domain is provided at page 27, lines 16-21, which relates to site-directed mutagenesis of the active site of the ketosynthase domain from an extension module from a cysteine to a glutamine residue. Significantly, there is no question that as of the priority date the methods of site-directed mutagenesis to insert a single amino acid mutation were well established and a skilled artisan would readily be able to generate such mutants. Thus, Applicants submit that a skilled artisan, apprised of the instant specification and the description therein of the mutated KS domains which are within the scope of the invention, would readily conclude that the Applicants had possession of the invention as of the priority date.

For all of the foregoing reasons, it is clear that in the present case, the Examiner has failed to satisfy the burden of proof with respect to the rejections based on lack of written description, as applied to the subject matter of claims 20, 23-25, 29, 30, and 43-63. These grounds of rejection should, therefore, be withdrawn.

CLAIMS 46, 48, 52, AND 53 ARE NOT ANTICIPATED BY KUHSTOSS ET AL.

The Examiner has rejected claims 46, 48, 52, and 53 under 35 U.S.C. §102(b) as allegedly anticipated by Kuhstoss et al. (Gene (1996) 183:231-236). The Examiner contends that Kuhstoss et al. teach a hybrid polyketide synthase comprising the loading module of the tylosin polyketide synthase and the extension modules of the spiramycin polyketide synthase.

Applicants continue to take issue with the Examiner's position for the reasons set forth in the Official Action response filed September 8, 2003. However, Applicants have cancelled claims 46, 48, 52, and 53, thereby obviating the instant rejection under 35 U.S.C. §102(b).

Applicants also submit that new claims 64-76 cannot reasonably be held to be anticipated by Kuhstoss et al. As noted by the Examiner, Kuhstoss et al. teach only a single hybrid polyketide synthase comprising the tylosin loading module and spiramycin extension modules. New claims 64-74 and 76, however, all recite loading modules which do not include the tylosin loading module. Claim 75, while drawn to a hybrid polyketide synthase comprising a tylosin loading module, specifically recites that the hybrid polyketide synthase produces only 12- or 14-membered macrolides. By contrast, Kuhstoss et al. teach making 16-membered macrolides only, as acknowledged by the Examiner at page 14 of the instant Official Action. Inasmuch as each and every aspect of the invention claimed in new claims 64-76 is neither taught nor suggested by Kuhstoss et al., Applicants submit that Kuhstoss et al. can not be considered to render the claimed subject matter unpatentable.

In view of the cancellation of claims 46, 48, 52, and 53, Applicants submit that the rejection under 35 U.S.C. §102(b) is untenable and respectfully request its withdrawal.

CLAIMS 20, 23-25, 29, 30, 43-45, 47, AND 49 ARE NOT RENDERED OBVIOUS BY U.S. PATENT 5,712,146 IN VIEW OF KHOSLA ET. AL.

The Examiner has rejected claims 20, 23-25, 29, 30, 43-45, 47 and 49 under 35 U.S.C. §103, as allegedly unpatentable over U.S. Patent 5,712,146 in view of Khosla et al. (Chemical Reviews (1997) 97:2577-2590). It is the Examiner's position that U.S. Patent 5,712,146 teaches the generation of a hybrid polyketide synthase by the combination of domains from various polyketide gene clusters. The Examiner further asserts that U.S. Patent 5,712,146 describes the spiramycin, erythromycin, and monensin polyketide synthases which purportedly contain a KSq domain, an AT domain which employs methylmalonyl-CoA, and an AT domain which contains an active-site arginine, respectively. Khosla et al. allegedly teach a 12-membered macrolide.

Applicants again respectfully disagree with the Examiner's position for the reasons set forth in the Official Action responses filed September 8, 2003 and November 4, 2002. Additionally, Applicants note that the Examiner relies on Khosla et al. "to support the reasonable expectation of success for the combinations suggested by [U.S. Patent 5,712,146]." Applicants reiterate that Khosla et al. teaches that ketosynthase domains with an active-site glutamine are "presumably inactive" (see page 2581, left column, second full paragraph). Thus, Khosla et al. provide absolutely no evidence of a "reasonable expectation of success" for employing ketosynthase domains with an active-site glutamine, in that such domains are indicated as being inactive. In the absence of any reasonable expectation of success properly evidenced by the prior art, there can clearly be no motivation or incentive for a skilled artisan to arrive at the instantly claimed invention, wherein the loading module contains a ketosynthase domain with an active-site glutamine. Thus, Applicants submit that a skilled artisan, apprised of U.S.

Patent 5,712,146 and the teaching of Khosla et al. that ketosynthase domains with an active-site glutamine presumably are inactive, would never arrive at the instantly claimed invention. Cf., In re Albrecht, 185 U.S.P.Q. 585 (CCPA 1975) (in view of clear indication of lack of utility in the prior art, the person of ordinary skill in the art lacks the necessary impetus to make the claimed subject matter).

There are additional reasons supporting Applicant's position that the subject matter of new claims 64-76 cannot be considered obvious over U.S. Patent 5,712,146 in view of Khosla et al. Indeed, the Examiner acknowledges at page 20 of the instant Official Action that claims 50, 51, 61, and 62, drawn to polyketide synthases comprising either the acyltransferase domain of module 6 of the niddamycin polyketide synthase or the acyltransferase domain module 4 of the FK506 polyketide synthase, are free of the prior art. Specifically, the Examiner concedes that "such exact combinations" cannot be rendered obvious by U.S. Patent 5,712,146. Accordingly, new claims 65 and 66, drawn to polyketide synthases comprising the specific acyltransferase domains stated above, are also free of the prior art.

Applicants also submit that claims 64 and 67-76 are free of the prior art because they are drawn to polyketide synthases comprising "exact combinations" of domains that cannot be rendered obvious by U.S. Patent 5,712,146. For example, claims 64 and 67-71 are all drawn to polyketide synthases wherein the acyltransferase domain of the loading module is selected from the group consisting of the acyltransferase domain from extension module 6 of the niddamycin polyketide synthase, extension module 4 of the FK506 polyketide synthase, extension module 2 of the rapamycin polyketide synthase, extension module 5 of the spiramycin polyketide synthase, and extension module 5 of the monensin polyketide synthase. Notably, U.S. Patent 5,712,146 is silent

as to incorporating these specific domains from an extension module into a loading module. It has long been recognized that silence in a reference is not a proper substitute for adequate disclosure of facts from which a conclusion of obviousness may justifiably follow. In re Burt, 148 U.S.P.Q. 548 (CCPA 1966).

As for claims 73-76, these claims are drawn to hybrid polyketide synthases wherein the loading domain is specifically identified as being from the monensin, oleandomycin, tylosin, and spiramycin polyketide synthases. A careful review plainly shows that U.S. Patent 5,712,146 fails to teach polyketide synthases specifically comprising such loading domains and therefore cannot render claims 73-76 obvious.

Lastly, claim 72 is drawn to a polyketides synthase wherein the KSq domain is the KSq domain from the oleandomycin loading module. Again, U.S. Patent 5,712,146 is silent with respect to such a specific combination of domains. See, In re Burt, 148 U.S.P.Q. 548 (CCPA 1966).

For all of the foregoing reasons, Applicants submit that claims 20, 23-25, 29, 30, 43-45, 47 and 49 and newly added claims 64-76 cannot be considered obvious over U.S. Patent 5,712,146 in view of Khosla et al. In an effort to expedite prosecution of the instant application, however, Applicants have cancelled claims 20, 23-25, 29, 30, 43-45, 47 and 49. Accordingly, the instant rejection is obviated and Applicants respectfully request its withdrawal.

CONCLUSION

In view of the amendments presented herewith, and the foregoing remarks, it is respectfully urged that the objections and rejections set forth in the December 16, 2003 Official Action be withdrawn and that this application be passed to issue.

In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone interview, the Examiner is requested to telephone the undersigned attorney at the phone number give below.

Respectfully submitted,
DANN, DORFMAN, HERRELL AND SKILLMAN
A Professional Corporation

By 
Patrick J. Hagan
PTO Registration No. 27,643

Telephone: (215) 563-4100
Facsimile: (215) 563-4044
Enclosure: Cane et al. (J. Am. Chem. Soc. (1993)115:522-526)

J. Am. Chem. Soc. 1993, *115*, 522–526

Macrolide Biosynthesis. 7. Incorporation of Polyketide Chain Elongation Intermediates into Methymycin

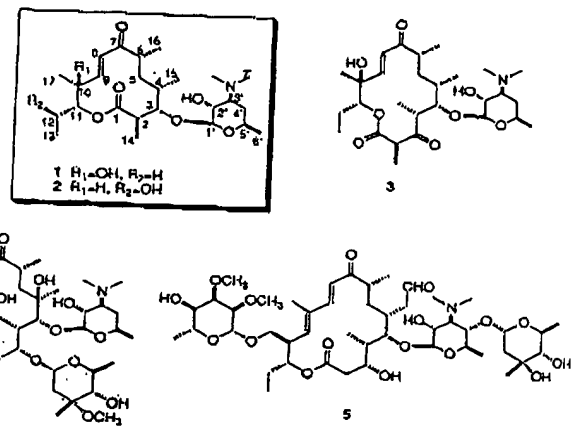
David E. Cane,* Ralph H. Lambalot, P. C. Prabhakaran, and Walter R. Ott

Contribution from the Department of Chemistry, Brown University, Providence, Rhode Island 02912. *Received July 13, 1992*

Abstract: Administration of [1-^{13}C]propionate to cultures of *Streptomyces venezuelae* SC 2366 gave methymycin (**1**), which was shown by ^{13}C NMR analysis to be labeled at the predicted sites, C-1, C-3, C-5, C-9, and C-11. Similarly, incorporation of [1,2-$^{13}C_2$]acetate gave methymycin labeled at C-7 and C-8. A series of presumptive intermediates of polyketide chain elongation was also successfully incorporated. Thus, feeding of (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl *N*-acetylcysteamine (NAC) thioester **7a** gave both methymycin (**1**) and neomethymycin (**2**) labeled as expected at C-10 and C-11. In a complementary experiment, (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester **7b** was incorporated into **1** and **2** without loss of deuterium. Finally, incorporation of (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoyl NAC thioester **10a** gave **1** and **2** labeled at C-8 and C-9. These results support a processive model of polyketide chain assembly in which the functionality and oxidation level are adjusted subsequent to each condensation step.

Methymycin (**1**) is a 12-membered macrolide antibiotic first isolated from *Streptomyces venezuelae* in 1954 by researchers at Squibb.[1a] Methymycin was the first macrolide to have its complete structure determined[1b] and the first to succumb to total synthesis.[1c] Methymycin is often accompanied by the cometabolite neomethymycin (**2**).[2] These metabolites show striking structural and stereochemical similarities to the 14-membered macrolides picromycin (**3**)[3] and erythromycin A (**4**) as well as the 16-membered macrolide tylosin (**5**), as first pointed out by Celmer (Chart I).[4] Extensive biosynthetic, enzymological, and molecular genetic investigations have established the polyketide origins of **4** and **5** and elucidated many of the details of the assembly of these functionally and stereochemically complex substances.[5] Significant new insights have recently come from cloning and sequencing and from partial expression of the structural genes encoding the formation of the parent erythromycin aglycone, 6-deoxyerythronolide B.[6] Two groups have independently demonstrated that the *eryA* gene is organized into three large (ca. 10 kb) open reading frames containing a series of domains apparently responsible for each of the microscopic steps of polyketide chain assembly and showing strong homology to analogous regions of fatty acid synthase genes. Remarkably, the organization of these domains appears to be collinear with the presumed order of the biochemical chain assembly steps. In the meantime, our own group and that of Hutchinson has demonstrated that it is possible to incorporate the *N*-acetylcysteamine (NAC) thioesters of putative intermediates of polyketide chain assembly intact into both erythromycin[7] and tylactone,[8] thereby providing strong biosynthetic evidence for the processive nature of reduced polyketide chain elongation. These techniques have subsequently been successfully applied by our own and several other groups in complementary studies of polyketide biosynthesis.[9] We now report the extension of these studies to the investigation of methymycin biosynthesis.

Chart I



Results

The 100.6-MHz ^{13}C NMR spectra of **1** and **2** were assigned using a combination of 1D and 2D NMR experiments including ^{13}C INEPT, ^{1}H COSY, and ^{1}H^{13}C HETCOSY (Table I). The lactonic (C-1, 175.2 ppm) and ketonic (C-7, 204.6 ppm) carbonyl signals for **1** were readily assigned on the basis of standard chemical shift parameters, as was the quaternary, hydroxyl-bearing carbon C-10 (74.1 ppm). The H-17 methyl protons appeared as a singlet at δ 1.35, and the N(CH$_3$)$_2$ signals at 2.27 were easily recognizable. The assignment of the corresponding ^{13}C NMR signals for C-17 (19.2 ppm) and N(CH$_3$)$_2$ (40.2 ppm) followed directly from the HETCOSY spectrum. The olefinic protons H-8

(1) (a) Isolation: Donin, M. N.; Pagano, J.; Dutcher, J. D.; McKee, C. M. *Antibiot. Annu.* **1953–54**, *1*, 179. Perlman, D.; O'Brien, E. *Antibiot. Chemother.* **1954**, *4*, 894. (b) Structure determination: Djerassi, C.; Zderic, J. A. *J. Am. Chem. Soc.* **1956**, *78*, 2907, 6390. Rickards, R. W.; Smith, R. M. *Tetrahedron Lett.* **1970**, 1025. (c) Synthesis: Masamune, S.; Kim, C. U.; Wilson, K. E.; Spessard, G. O.; Georghiou, P. E.; Bates, G. S. *J. Am. Chem. Soc.* **1975**, *97*, 3512. Masamune, S.; Yamamoto, H.; Kamata, S.; Fukuzawa, A. *J. Am. Chem. Soc.* **1975**, *97*, 3513.

(2) Djerassi, C.; Halpern, O. *Tetrahedron* **1958**, *3*, 255.

(3) Cultures of *S. venezulae* also produce narbonolide, a precursor of picromycin: Hori, T.; Maezawa, I.; Nagahama, N.; Suzuki, M. *J. Chem. Soc., Chem. Commun.* **1971**, 304.

(4) Celmer, W. D. *J. Am. Chem. Soc.* **1965**, *87*, 1801. Celmer, W. D. *Pure Appl. Chem.* **1971**, *28*, 413. Cane, D. E.; Celmer, W. D.; Westley, J. W. *J. Am. Chem. Soc.* **1983**, *105*, 3594.

(5) Omura, S.; Tanaka, Y. In *Macrolide Antibiotics: Chemistry, Biology, and Practice*; Omura, S., Ed.; Academic Press: New York, 1984; pp 199–299. Staunton, J. *Angew. Chem., Intl. Ed. Engl.* **1991**, *30*, 1302. Baltz, R. H.; Seno, E. T. *Antimicrob. Agents Chemother.* **1981**, *120*, 214. Fishman, S. E.; Cox, K.; Larson, J. L.; Reynolds, P. A.; Seno, E. T.; Yeh, W.-K.; Van Frank, R.; Hershberger, C. L. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8248.

(6) (a) Donadio, S.; Katz, L. *Gene* **1992**, *111*, 51. Donadio, S.; Staver, M. J.; Mcalpine, J. B.; Swanson, S. J.; Katz, L. *Science* **1991**, *252*, 675. (b) Bevitt, D. J.; Cortes, J.; Haydock, S. F.; Leadlay, P. F. *Eur. J. Biochem.* **1992**, *204*, 39. Caffrey, P.; Green, B.; Packman, L. C.; Rawlings, B. J.; Staunton, J.; Leadlay, P. E. *Eur. J. Biochem.* **1991**, *195*, 823. Cortes, J.; Haydock, S. F.; Roberts, G. A.; Bevitt, D. J.; Leadlay, P. F. *Nature* **1990**, *348*, 176.

(7) (a) Cane, D. E.; Yang, C. *J. Am. Chem. Soc.* **1987**, *109*, 1255. (b) Cane, D. E.; Yang, C.; Ott, W. R. *Proceedings of the Seventh International Symposium on Biology of Actinomycetes*; Japan Antibiotics Research Association: Tokyo, 1988; pp 395–400. (c) Cane, D. E.; Prabhakaran, P. C.; Tan, W.; Ott, W. R. *Tetrahedron Lett.* **1991**, *32*, 5457.

(8) Yue, S.; Duncan, J. S.; Yamamoto, Y.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1987**, *109*, 1253.

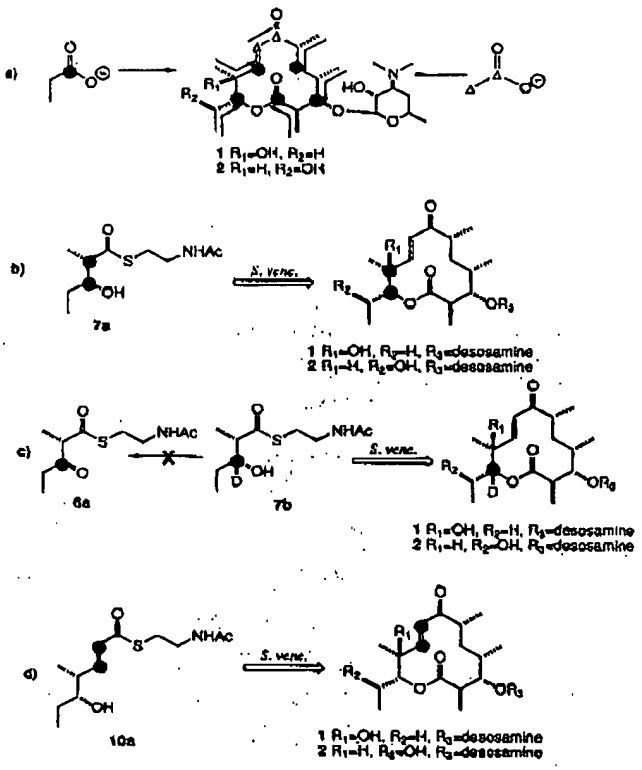
(9) (a) Cane, D. E.; Ott, W. R. *J. Am. Chem. Soc.* **1988**, *110*, 4840. Cane, D. E.; Tan, W.; Oh, W. R. *J. Am. Chem. Soc.*, following article in this issue. (b) Spavold, Z. M.; Robinson, J. A. *J. Chem. Soc., Chem. Commun.* **1988**, 4. (c) Li, Z.; Martin, M.; Vederas, J. C. *J. Am. Chem. Soc.* **1992**, *114*, 1531. Yoshizawa, Y.; Li, Z.; Rosse, P. B.; Vederas, J. C. *J. Am. Chem. Soc.* **1990**, *112*, 3212. (d) Staunton, J.; Sutkowski, A. C. *J. Chem. Soc., Chem. Commun.* **1991**, 1110. Jacobs, A.; Staunton, J.; Sutkowski, A. C. *J. Chem. Soc., Chem. Commun.* **1991**, 1113.

0002-7863/93/1515-522$04.00/0

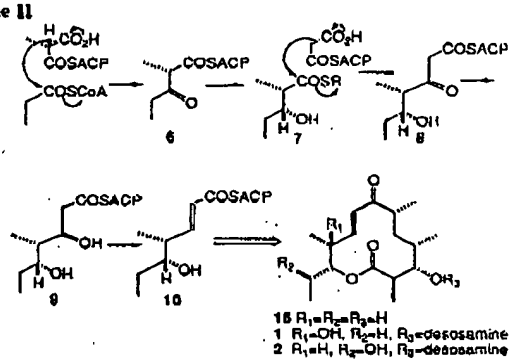
Table I. ^1H (400 MHz) and ^{13}C (100.6 MHz) NMR Spectral ($CDCl_3$) Data of Methymycin (1) and Neomethymycin (2)

1				2			
H	δ (m, *J*(Hz), area)	C	δ (m)	H	δ (m, *J* (Hz), area)	C	δ (m)
2	2.87 (dq, 6.9, 10.5, 1 H)	1	175.2 (s)	2	2.88 (dq, 6.9, 10.5, 1 H)	1	174.8 (s)
3	3.60 (d, 10.5, 1 H)	2	44.2 (d)	3	3.59 (d, 10.4, 1 H)	2	43.9 (d)
4	1.25 (m)	3	85.5 (d)	4	1.25 (m)	3	85.6 (d)
5a	1.75 (m)	4	33.6 (d)	5a	1.68 (m, 1 H)	4	33.4 (d)
5b	1.50 (m)	5	33.9 (t)	5b	1.4 (m, 1 H)	5	34.1 (t)
6	2.5 (m, 1 H)	6	45.1 (d)	6	2.52 (m, 1 H)	6	45.1 (d)
8	6.34 (d, 15.9, 1 H)	7	204.6 (s)	8	6.44 (dd, 1.3, 15.7, 1 H)	7	205.2 (s)
9	6.60 (d, 15.9, 1 H)	8	125.6 (d)	9	6.76 (dd, 5.5, 15.7, 1 H)	8	126.2 (d)
11	4.75 (dd, 10.8, 2.2, 1 H)	9	148.9 (d)	10	3.05 (m, 1 H)	9	147.1 (d)
12a	1.95 (m, 2.2, 7.2, 1 H)	10	74.1 (s)	11	4.79 (dd, 2.3, 9.0, 1 H)	10	35.4 (d)
12b	1.5 (m, 1 H)	11	76.3 (d)	12	3.89 (dq, 6.2, 9.0, 1 H)	11	75.4 (d)
13	0.90 (t, 7.4, 3 H)	12	21.2 (t)	13	1.20 (d, 6.2, 3 H)	12	66.4 (d)
14	1.44 (d, 7.0, 3 H)	13	10.6 (q)	14	1.41 (d, 7.0, 3 H)	13	21.0 (q)
15	1.02 (d, 6.7, 3 H)	14	16.1 (q)	15	1.02 (d, 6.7, 3 H)	14	15.9 (q)
16	1.17 (d, 7.0, 3 H)	15	17.3 (q)	16	1.19 (d, 7.0, 3 H)	15	17.4 (q)
17	1.35 (s, 3 H)	16	17.5 (q)	17	1.16 (d, 6.8, 3 H)	16	17.6 (q)
1′	4.24 (d, 7.3, 1 H)	17	19.2 (q)	1′	4.24 (d, 7.3, 1 H)	17	9.8 (q)
2′	3.22 (dd, 7.3, 10.2, 1 H)	1′	105.0 (d)	2′	3.23 (dd, 7.3, 10.2, 1 H)	1′	105.1 (d)
3′	2.5 (m, 1 H)	2′	70.3 (d)	3′	2.52 (m, 1 H)	2′	70.3 (d)
4a′	1.65 (m, 1 H)	3′	65.8 (d)	4a′	1.68 (m, 1 H)	3′	65.9 (d)
4b′	1.2 (m, 1 H)	4′	28.3 (t)	4b′	1.2 (m, 1 H)	4′	28.3 (t)
5′	3.5 (m, 1 H)	5′	69.4 (d)	5′	3.48 (m, 1 H)	5′	69.5 (d)
6′	1.23 (d, 6.1, 3 H)	6′	21.1 (q)	6′	1.23 (d, 6.1, 3 H)	6′	21.1 (q)
7′	2.27 (s, 6 H)	7′	40.2 (q)	7′	2.28 (s, 6 H)	7′	40.2 (q)

Scheme I



Scheme II



and H-9 appeared as components of an AB quartet at δ 6.34 and 6.60, respectively, correlated with the corresponding ^{13}C NMR signals at 125.6 (C-8) and 148.9 ppm (C-9). The assignments of the remaining macrolide and desosaminyl[10] ^1H and ^{13}C NMR signals were completed in a straightforward manner and are summarized in Table I along with the corresponding assignments for neomethymycin (2).

Preliminary incorporation experiments confirmed the expected polyketide origin of methymycin.[11,12] Administration of [1-^{13}C]propionate to cultures of *Streptomyces venezuelae* SC2366 gave methymycin (1) which was shown by ^{13}C NMR analysis to be labeled at the predicted sites (C-1, C-3, C-5, C-9, C-11) (20 excess atom % ^{13}C) (Scheme Ia). Similar feeding of sodium [1,2-$^{13}C_2$]acetate gave rise to the expected enhanced and coupled doublets (J_{CC} = 50.3 Hz) corresponding to C-7 and C-8. Thus methymycin was shown to be derived from five propionates and one acetate precursor.

According to the emerging picture of polyketide chain elongation, formation of the macrolide aglycone of methymycin should be initiated by condensation of a propionyl thioester starter unit with an equivalent of (2*R*)-methylmalonyl CoA[13] (as the enzyme-bound ACP thioester) (Scheme II). Reduction of the resulting β-keto ester 6 will give (2*S*,3*R*)-2-methyl-3-hydroxypentanoate as the enzyme-bound thioester 7. Indeed, this same intermediate has already been implicated in the formation of both erythromycin and nargenicin.[7,9a] Initial attempts at intact in-

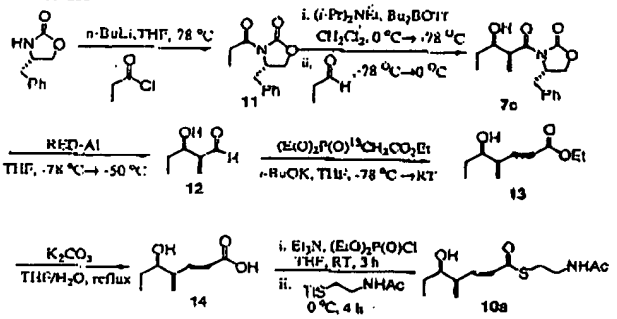
(10) The desosaminyl assignments were in close agreement with those reported for the desosamine moiety of erythromycin A: Everett, J. R.; Tyler, J. W. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2599.

(11) Birch et al. have reported incorporation experiments with ^{14}C-labeled precursors which support the derivation of methymycin from five propionates and one acetate: Birch, A. J.; Djerassi, C.; Dutcher, J. D.; Major, J.; Perlman, D.; Pride, E.; Rickards, R. W.; Thomson, P. J. *J. Chem. Soc.* **1964**, 5274. Birch, A. J.; Pride, E.; Rickards, R. W.; Thomson, P. J.; Dutcher, J. D.; Perlman, D.; Djerassi, C. *Chem. Ind. (London)* **1960**, 1245.

(12) Biosynthetic studies on the closely related macrolide picromycin (3) have been reported: Omura, S.; Takeshima, H.; Nakagawa, A.; Miyazawa, J. *J. Antibiot.* **1976**, *29*, 316.

(13) Cane, D. E.; Liang, T.; Taylor, P. B.; Chang, C.; Yang, C. *J. Am. Chem. Soc.* **1986**, *108*, 4957. Hutchinson, C. R.; Sherman, M. M.; McInnes, A. G.; Walter, J. A.; Vederas, J. C. *J. Am. Chem. Soc.* **1981**, *103*, 5956. Sood, G. R.; Robinson, J. A.; Ajaz, A. A. *J. Chem. Soc., Chem. Commun.* **1984**, 1421.

Scheme III



corporation of the dipropionate intermediate as the corresponding NAC thioester, (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl NAC thioester **7a**, prepared as previously described,[7,9a] proved difficult, resulting largely in degradation of the precursor with no detectable enrichment of the macrolide products. After extensive experimentation, it was eventually found that successful incorporation of **7a** could be achieved by the use of replacement cultures of *S. venezuelae* ATCC 15439 in combination with careful monitoring of the timing of macrolide production, using the UV absorption maximum at λ_{225} (ϵ = 10 500, MeOH) to estimate the macrolide content of $CHCl_3$ extracts of the fermentation broth. It was found to be critical that the cells of *S. venezuelae* be harvested and resuspended in replacement medium immediately following the onset of macrolide production. Both methymycin (**1**) (J_{CC} = 43.0 Hz) and neomethymycin (**2**) (J_{CC} = 36.5 Hz) obtained from the feeding of **7a** (30 mg/100 mL of culture) exhibited the expected pair of enhanced and coupled doublets corresponding to C-10 and C-11 in their respective ^{13}C NMR spectra (0.15 atom excess % ^{13}C) (Scheme Ib).

In order to rule out the possibility that incorporation of **7a** involves initial reoxidation to the corresponding β-keto ester **6a**, we next prepared (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester **7b**[7b] which was fed (120 mg) to a 100-mL replacement culture of *S. venezuelae* supplemented with 4-pentynoic acid[9c] in order to suppress degradation of the substrate by β-oxidation. The resulting samples of methymycin and neomethymycin each carried both ^{13}C and deuterium at C-11 (0.1 atom % excess), as evidenced by the appearance of the characteristic isotope-shifted triplet in the individual ^{13}C NMR spectra (**1**, $\Delta\delta$ = 0.40 ppm, J_{CD} = 17.3 Hz; **2**, $\Delta\delta$ = 0.35 ppm, J_{CD} = 20.9 Hz) (Scheme Ic). In each case, the triplet collapsed to a singlet upon broad-band deuterium decoupling ($^{13}C\{^2H,^1H\}$ NMR). Again, completely consistent results have previously been reported for the intact incorporation of **7b** into both erythromycin and nargenicin.[7c]

Having established the ability of the dipropionate intermediate to serve as a substrate in whole-cell feeding experiments, we turned our attention to the presumptive triketide intermediate, (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptanoyl NAC thioester **10a**, which was readily prepared in diastereomerically pure form (Scheme III). The synthetic procedures previously developed[7,9a,14] for the preparation of **7a** were used to obtain (4*S*,2′*S*,3′*R*)-[1′-^{13}C]-3-(2′-methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone (**7c**), which was readily reduced with sodium bis(2-methoxyethoxy)aluminum hydride[15] (RED-Al) in THF to give the corresponding 3-hydroxy aldehyde **12**. Emmons reaction of **12** with triethyl [2-^{13}C]phosphonoacetate in THF using potassium *tert*-butoxide as the base and hydrolysis of the resulting [2,3-$^{13}C_2$]-unsaturated ester **13** with K_2CO_3 in aqueous THF gave the corresponding acid **14**. Finally, **14** was converted to the desired NAC thioester **10a** by treatment with diethyl chlorophosphonate followed by thallous *N*-acetylcysteamine.[16,17] Feeding of **10a** (50 mg) to replacement cultures of *S. venezuelae* gave labeled **1** and **2** (0.5 atom % excess), which exhibited the expected pairs of enhanced and coupled doublets for C-8 and C-9 in the ^{13}C NMR spectrum (**1**, J_{CC} = 70.5 Hz; **2**, J_{CC} = 70.9 Hz) (Scheme Id).

Discussion

The results reported here complement and extend previous reports of the incorporation of intermediates of polyketide chain elongation into the closely related macrolides erythromycin (**4**)[7] and tylosin (**5**),[8] as well as the antibiotic nargenicin.[7b,c,9a] Consistent with the findings of Vederas,[9c] we have observed that precise timing of the administration of labeled precursors and control of unwanted substrate degradation through the use of replacement cultures in combination with appropriate inhibitors of fatty acid β-oxidation can be critical to the successful incorporation of NAC thioesters. In addition, the use of ^{13}C doubly labeled substrates not only allows direct detection of the intact incorporation of precursors but conveniently extends the useful range of reliable detection to 0.1–0.2 atom % excess ^{13}C enrichment.

Taken together, these incorporation studies are fully consistent with a processive model of polyketide chain elongation in which the oxidation level and stereochemistry of the growing polyketide chain are adjusted subsequent to each individual condensation step[6-8] (Scheme II). Interestingly, methymycin (**1**), neomethymicin (**2**), and erythromycin (**4**), as well as nargenicin, all share a common precursor, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl thioester **7**,[7,9a] whereas tylosin is derived from the corresponding (2*R*,3*R*) diastereomer of **7**.[8] The pathways to these macrolides apparently diverge at this point, with **7** undergoing condensation with an enzyme-bound derivative of malonyl CoA (Scheme II) on the way to **1** and **2**, while condensation with methylmalonyl CoA is required for the formation of both erythromycin and nargenicin. The successful incorporation of the triketide substrate, (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoyl NAC thioester **10a**, into both methymycin (**1**) and neomethymycin (**2**) is fully consistent with the proposed intermediacy of **10**, presumably generated from **7** by a sequence of malonyl condensation, keto reduction, and dehydration, as illustrated in Scheme II. In further support of these ideas, we have recently isolated the presumptive product of the polyketide synthase 10-deoxymethynolide (**15**), the parent aglycone of the methymycin family of antibiotics.[18]

The successful incorporation of intermediates of polyketide chain elongation into a variety of macrolides and other polyketide natural products[7-9] assumes particular significance in light of the enormously important molecular genetic discoveries of the groups at Abbott Laboratories[6a] and Cambridge[6b] regarding the organization of the *eryA* gene. Solely on the basis of the inferred organization of the gene product, with each catalytic domain presumably being responsible for individual steps of polyketide chain elongation and functional group modification, one might be tempted to conclude that the structure of the eventually formed 6-deoxyerythronolide B, or by logical extension any macrolide aglycone, would be exclusively the result of the organization of the polyketide synthase. According to this simplified picture, each module of enzymes responsible for a round of condensation and appropriate reductions, dehydrations, and reductions simply accepts as substrate the product of the neighboring module. The finding that it is possible to incorporate intact a number of advanced intermediates of polyketide chain elongation into a variety of macrolides indicates that the actual macrolide biosynthetic mechanism is considerably more sophisticated. The fact that each advanced precursor is properly processed by the polyketide synthase during incorporation experiments with intact cells indicates that there is a significant element of molecular recognition involved in the operation of each biosynthetic module. Which structural features of each intermediate are most critical to proper recognition and whether this molecular recognition involves the acyltransferase, keto synthase,

(14) Evans, D. A.; Bartroli, J.; Shih, T. L. *J. Am. Chem. Soc.* **1981**, *103*, 2127. Evans, D. A.; Ennis, M. D.; Le, T.; Mandel, N.; Mandel, G. *J. Am. Chem. Soc.* **1984**, *106*, 1154. Evans, D. A.; Bitton, T. C.; Ellman, J. A. *Tetrahedron Lett.* **1987**, *28*, 6141.

(15) Meyers, A. I.; Spohn, R. F.; Linderman, R. J. *J. Org. Chem.* **1985**, *50*, 3633.

(16) Schwab, J. M.; Klassen, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 7217.

(17) Masamune, S.; Kamata, S.; Diakur, J.; Sugihara, Y.; Bates, G. S. *Can. J. Chem.* **1975**, *53*, 3693.

(18) Lambalot, R. H.; Cane, D. E. *J. Antibiot.*, In press.

ACP domains, or some combination of all three remain to be established. Further investigations of the organization and control of this complex and intriguing biosynthetic process are in progress.

Experimental Section

Materials and Methods. All reactions were run under argon atmosphere using oven-dried syringes and glassware when appropriate. THF and CH_2Cl_2 were distilled from Na/benzophenone. Et_2O was distilled from P_2O_5. ^1H (250 or 400 MHz) and ^{13}C (100.6 MHz) NMR spectra were recorded on Bruker WM-250 and AM-400 NMR spectrometers. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR spectrophotometer. Optical rotation measurements were obtained using a Perkin-Elmer 241 polarimeter. UV/vis spectra were obtained using a Perkin-Elmer 552A spectrophotometer. Mass spectra were obtained by chemical ionization with NH_4^+ or electron ionization using a Kratos MS80RFA mass spectrometer. Reagents and solvents were obtained from Aldrich Chemical Co., Cambridge Isotope Laboratories, and Los Alamos National Laboratories. (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-Methyl-3-hydroxypentanoyl NAC thioester **7a**[7,9a] and (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester **7b**[7b] were prepared as previously described. *Streptomyces venezuelae* SC2366 was a gift from the Squibb Institute for Medical Research. *Streptomyces venezuelae* ATCC 15439 was received as a freeze-dried pellet. All culture media and glassware were autoclaved prior to use, and all biological manipulations were conducted under a Labconco sterile hood enclosure. Nanopure water obtained from a Barnstead Nanopure II water purification system was used to make all culture media. All *S. venezuelae* cultures were grown at 27 °C and 250 rpm in a New Brunswick Scientific floor model shaker. A Sorvall RC-5 centrifuge equipped with a GSA rotor was used for all harvesting and replacement procedures.

Cultivation of *Streptomyces venezuelae* ATCC 15439 for Advanced Precursor Feeding Experiments. An autoclaved 100-mL seed medium (20 g of glucose, 15 g of soybean flour, 5 g of $CaCO_3$, 1 g of NaCl, and 0.002 g of $COCl_2{\cdot}6H_2O$ per 1 L of nanopure H_2O, pH adjusted to 7.2 with 2 N NaOH) in a 500-mL baffled flask equipped with a sponge stopper was inoculated with a scraping from an agar slant of *S. venezuelae* ATCC 15439. The culture was grown on a shaker at 27 °C and 250 rpm for 48 h. Vegetative cultures (100 mL) were initiated by inoculation of the vegetative medium (20 g of glucose, 30 g of soybean flour, 2.5 g of $CaCO_3$, 1 g of NaCl, and 0.002 g of $COCl_2{\cdot}6H_2O$ per 1 L of nanopure H_2O, pH adjusted to 7.2 with 2N NaOH) with 3 mL of seed culture and grown under the same conditions. At 18 h the vegetative culture was transferred into an autoclaved 250-mL centrifuge bucket and centrifuged at 10000 rpm (Sorvall GSA rotor) for 15 min. The mycelia were resuspended in 100 mL of replacement medium (100 g of glucose, 2.5 g of $CaCO_3$, 1 g of NaCl, and 0.002 g of $COCl_2{\cdot}6H_2O$ per 1 L of nanopure H_2O, pH adjusted to 7.2 with 2 N NaOH) and decanted into an autoclaved 500-mL baffled flask. Isotopically labeled substrates (25–120 mg) were administered in 1 mL of 100% EtOH, and the culture was grown on a shaker at 27 °C and 250 rpm for 72 h.

UV Assay for Monitoring Combined Methymycin and Neomethymycin Production. A 1.5-mL sample was withdrawn from an actively fermenting culture and centrifuged (5 min at 14000 rpm) in a 1.5-mL Eppendorf tube. The supernatant was then decanted into a 13 × 100 mm test tube and basified to pH > 9 (2 drops of 2 N NaOH). The broth was extracted with 2 × 1.5 mL of $CHCl_3$ by gently agitating to avoid an emulsion. Any emulsion formed could be removed by centrifugation for 10 min at 5000 rpm. The organic extract was transferred to a clean dry test tube via a Pasteur pipet and the solvent was removed by rotary evaporation. A gentle stream of nitrogen was then passed through the test tube to remove any traces of $CHCl_3$ from the residue. Spectroscopic grade MeOH (5 mL) was added via volumetric pipet, and the solution was agitated to dissolve the residue. The absorbance of the crude extract was then measured at 225 nm (ϵ = 10500).

Isolation and Purification of Methymycin and Neomethymycin. The vegetative replacement culture was harvested by pouring the 100 mL of fermentation broth into a 250-mL centrifuge bucket and centrifuging for 15 min at 10000 rpm (Sorvall GSA rotor). The supernatant was decanted into a 500-mL flask and the pellet resuspended in 100 mL of acetone. The acetone wash was centrifuged for 15 min at 10000 rpm and the supernatant decanted into a 200-mL round-bottom flask. The solvent was removed by rotary evaporation, and the remaining yellow extract was washed into the first supernatant. The pH of the combined mixtured was adjusted to 9.5 with 2 N NaOH, and the aqueous phase was extracted with 4 × 50 mL of $CHCl_3$. The $CHCl_3$ extract was dried with Na_2SO_4 and the solvent removed by rotary evaporation followed by drying in vacuo to an amber oil (15 mg/100 mL). The crude mixture was purified by flash chromatography on silica gel (10 × 130 mm) with 90% $CHCl_3$/9.9% MeOH/0.1% NH_4OH. Cleaner separations were achieved by slowly ramping the MeOH from 0% to 10%. Fractions were monitored by silica TLC followed by developing with vanillin stain (0.75% vanilla, 1.5% H_2SO_4, MeOH). Methymycin had the higher R_f (0.24) and stained dark brown with vanillin, while neomethymycin had the lower R_f (0.18) and stained orange with vanillin.

(4*S*)-3-[1′-^{13}C]Propionyl-4-benzyl-2-oxazolidinone (11). (4*S*)-3-[1′-^{13}C]Propionyl-4-benzyl-2-oxazolidinone (**11**) was prepared from (4*S*)-4-benzyl-2-oxazolidinone following the previously described procedure:[7,14] mp = 44–45 °C; IR ($CHCl_3$) ν 3025, 1781, 1662, 1387, 1078, 1011 cm^{-1}; 400-MHz ^{1}H NMR ($CDCl_3$) δ 7.34–7.19 (m, 5 H, C_6H_5), 4.66 (m, 1 H, NC*H*), 4.17 (m, 2 H, CH_2O), 3.28 (dd, J = 3.2, 13.4 Hz, 1 H, $CHHC_6H_5$), 3.01–2.87 (m, 2 H, CH_3CH_2), 2.77 (dd, J = 9.6, 13.4 Hz, 1 H, $CHHC_6H_5$), 1.18 (dt, J = 5.56, 7.32 Hz, 3 H, CH_3CH_2); 100-MHz ^{13}C NMR ($CDCl_3$) δ 173.9 (enriched), 153.4, 135.3, 129.3, 128.8, 127.2, 66.0, 55.0, 37.3, 29.0 (d, J = 50 Hz), 8.1; $[\alpha]_D$ = +59.19° ($CHCl_3$, l = 1.0 dm, 0.016 g/mL); EIMS M^+ 234 *m/e*.

(4*S*,2′*S*,3′*R*)-3-(2′-Methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone (7c).[7,14] To a stirred solution (0.4 M) of unlabeled **11** (1.0 g, 4.29 mmol) in CH_2Cl_2 at 0 °C (ice bath) in a 100-mL, 2-neck, RB flask under argon atmosphere were added 894 μL of diisopropylethylamine (1.2 equiv) and 4.7 mL of dibutylboron triflate (1.0 M in CH_2Cl_2, 1.1 equiv). The mixture was allowed to stir at 0 °C for 30 min before cooling to −78 °C (dry ice/acetone) and stirring for another 30 min. Following the addition of 340 μL of propionaldehyde (neat, 1.1 equiv) the reaction was stirred for 30 min at −78 °C, after which time the dry ice bath was removed and the reaction allowed to warm to room temperature for 1.5 h. The reaction was cooled to 0 °C and quenched with 20 mL of MeOH and 10 mL of pH 7.4 buffered water, and the borate was oxidized with 10 mL of 30% H_2O_2 while stirring for 1 h. The crude was extracted with 3 × 25 mL of CH_2Cl_2 and purified by flash silica gel chromatography (10% EtOAc/$CHCl_3$) to yield 747 mg (60%) of white crystalline product: R_f = 0.35 (10% EtOAc/$CHCl_3$); mp = 79–81 °C; IR ($CHCl_3$) ν 3530, 3019, 2879, 1781, 1686, 1387 cm^{-1}; 400-MHz ^{1}H NMR ($CDCl_3$) δ 7.36–7.20 (m, 5 H, aromatic H's), 4.73–4.69 (m, 1 H, NC*H*), 4.25–4.17 (m, 2 H, CH_2O), 3.87 (m, 1 H, C*H*OH), 3.79 (dq, J = 2.7, 7.04 Hz, 1 H, C*H*CH$_3$), 3.26 (dd, J = 3.35, 13.41 Hz, 1 H, C*H*HPh), 2.87 (b, 1 H, O*H*), 2.79 (dd, J = 9.43, 13.40 Hz, 1 H, CH*H*Ph), 1.58–1.47 (m, 2 H, CH_3CH_2), 1.25 (d, J = 7.02 Hz, 3 H, $CHCH_3$), 0.98 (t, J = 7.4 Hz, 3 H, CH_3CH_2); 100-MHz ^{13}C NMR ($CDCl_3$) δ 177.6, 153.0, 135.0, 129.4, 129.0, 127.4, 73.0, 66.2, 55.1, 41.7, 37.8, 26.8, 10.4, 10.3; $[\alpha]_d$ = +51.47° ($CHCl_3$, l = 1.0 dm, 0.0293 g/mL); CIMS (NH_4^+) (M + H)$^+$ 292 *m/e*.

(4*S*,2′*S*,3′*R*)-[1′-^{13}C]-3-(2′-Methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone (7c). (4*S*,2′*S*,3′*R*)-[1′-^{13}C]-3-(2′-Methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone ([1′-^{13}C]-**7c**) was prepared from (4*S*)-3-[1′-^{13}C]propionyl-4-benzyl-2-oxazolidinone following the same procedure described for unlabeled **7c**: mp = 81.5–82 °C; IR ($CHCl_3$) ν 3482, 2970, 2878, 1779, 1649, 1455, 1386, 1213, 1112, 969, 757 cm^{-1}; 400-MHz ^{1}H NMR ($CDCl_3$) δ 7.34–7.19 (m, 5 H, aromatic H's), 4.70 (m, 1 H, NC*H*), 4.23–4.15 (m, 2 H, CH_2O), 3.86–3.77 (m, 2 H, C*H*OH, C*H*CH$_3$), 3.23 (dd, J = 3.1, 13.4 Hz, 2 H, C*H*HPh, CHO*H*), 2.80 (dd, J = 9.3, 13.4 Hz, 1 H, CH*H*Ph), 1.6–1.4 (m, 2 H, CH_3CH_2), 1.25 (dd, J = 5.39, 6.68 Hz, 3 H, $CHCH_3$), 0.97 (t, J = 7.35 Hz, 3 H, CH_3CH_2); 100-MHz ^{13}C NMR ($CDCl_3$) δ 177.1 (^{13}C enriched), 152.9 (d, J_{CC} = 4.5 Hz), 134.9, 129.2, 128.7, 127.2, 70.0 (d, J_{CC} = 2.2 Hz), 54.9, 41.6 (d, J_{CC} = 49.3 Hz), 37.5, 26.7 (d, J_{CC} = 3.6 Hz), 10.2; $[\alpha]_D$ = 48.46 ($CHCl_3$, l = 1.0 dm, 0.1881 g/mL); CIMS (NH_4^+) (M + H)$^+$ 293 *m/e*.

(2*S*,3*R*)-2-Methyl-3-hydroxypentanal (12).[15] A 100-mL, 2-neck, RB flask equipped with an argon inlet, septum, and stir bar was charged with 60 mL of dry THF and 1.30 mL of RED-Al (3.4 M in toluene, 4.4 mmol, 1.1 equiv) under an argon atmosphere. The solution was then cooled to −78 °C, and 1.168 g of unlabeled **7c** (4.0 mmol) in 10 mL of THF was added slowly via cannula. The evolution of gas could be seen as the solution was stirred for 10–15 min at −78 °C. The reaction was then warmed to −50 °C ($CHCl_3$/CO_2) and stirred between −55 and −40 °C for 1 h. The reaction was quenched at −50 °C with 12 mL of EtOAc and 3 mL of MeOH and then poured into a mixture of 25 mL of 1 M HCl (saturated with NaCl) and 45 mL of Et_2O and stirred at −20 °C for 10–15 min. The aqueous layer froze out as a gel. The ethereal layer was decanted and the aqueous layer rinsed quickly with 2 × 30 mL of Et_2O. The combined organic extracts were dried over K_2CO_3 and concentrated to 970 mg of oil. The crude aldehyde was dried in vacuo and taken immediately on to the Wittig reaction.

(2*S*,3*R*)-[1-^{13}C]-2-Methyl-3-hydroxypentanal (12). (2*S*,3*R*)-[1-^{13}C]-2-Methyl-3-hydroxypentanal ([1-^{13}C]-**12**) was prepared from (4*S*,2′*S*,3′*R*)-[1′-^{13}C]-3-(2′-methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone following the same procedure as described for unlabeled **12**.

Ethyl (4*R*,5*R*)-4-Methyl-5-hydroxy-2-heptenoate (13).[15] To 50 mL of dry THF in a 100-mL flask under argon was added 912 μL of triethyl phosphonoacetate (4.6 mmol, 1.15 equiv), followed by 493 mg of potassium *tert*-butoxide (4.4 mmol, 1.1 equiv). The mixture was stirred at room temperature for 5 min before cooling to −78 °C (dry ice/acetone).

The crude β-hydroxy aldehyde (12) was added in 5 mL of THF and stirred overnight while warming to room temperature. The mixture was poured into 5 mL of brine, extracted with 3 × 30 mL of Et_2O, dried over K_2CO_3, and concentrated in vacuo to 1.12 g of oil (151%). Flash chromatography (60% Et_2O/hexanes) provided 165 mg of pure product as an oil (22%): R_f = 0.31 (60% Et_2O/hexanes); IR (neat) ν 3459, 2969, 1714, 1651, 1462, 1370, 1278, 1184, 1041, 985, 868 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 6.95 (dd, J = 7.8, 15.8 Hz, 1 H, C*H*=CHCO$_2$), 5.86 (dd, J = 1.25, 15.7 Hz, 1 H, CH=C*H*CO$_2$), 4.19 (q, J = 7.1 Hz, 2 H, OC*H*$_2$CH$_3$), 3.49 (m, 1 H, C*H*OH), 2.43 (m, 1 H, C*H*CH$_3$), 1.92 (b, 1 H, O*H*), 1.5 (m, 1 H, CH$_3$C*H*H), 1.4 (m, 1 H, CH$_3$CH*H*), 1.29 (t, J = 7.1 Hz, 3 H, OCH$_2$C*H*$_3$), 1.09 (d, J = 6.8 Hz, C*H*$_3$CH), 0.97 (t, J = 7.4 Hz, 3 H, C*H*$_3$CHH); 100-MHz ^{13}C NMR ($CDCl_3$) δ 166.6, 151.1, 121.5, 75.9, 60.3, 42.2, 27.3, 14.2, 13.9, 10.2; $[\alpha]_D$ = +35.16° ($CHCl_3$, l = 1.0 dm, 0.0062 g/mL); CIMS (NH_4^+) (M + H)$^+$ 187 m/e; exact mass calcd for $C_{10}H_{19}O_3$ 187.1334, found 187.1327.

Ethyl (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-Methyl-5-hydroxy-2-heptenoate (13). Ethyl (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoate ([2,3-$^{13}C_2$]-13) was prepared from (2*S*,3*R*)-[1-^{13}C]-2-methyl-3-hydroxypentanal and triethyl [2-^{13}C]phosphonoacetate following the same procedure described for unlabeled 13: IR (neat) ν 3459, 2971, 2878, 1708, 1598, 1459, 1274, 1183, 979, 756 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 6.95 (dddd, J = 1.93, 7.84, 15.77, 154.28 Hz, 1 H, *H*^{13}C=^{13}CHCO$_2$), 5.86 (ddd, J = 0.77, 15.77, 162.03 Hz, 1 H, H^{13}C=^{13}C*H*CO$_2$), 4.19 (q, J = 7.13 Hz, 2 H, CO$_2$C*H*$_2$CH$_3$), 3.49 (m, 1 H, C*H*OH), 2.44 (m, 1 H, C*H*CH$_3$), 1.9 (b, 1 H, O*H*), 1.60–1.50 (m, 1 H, CH$_3$C*H*H), 1.46–1.36 (m, 1 H, CH$_3$CH*H*), 1.29 (t, J = 7.13 Hz, 3 H, CO$_2$CH$_2$C*H*$_3$), 1.09 (dd, J = 4.90, 6.75 Hz, 3 H, CHC*H*$_3$), 0.97 (t, J = 7.40 Hz, 3 H, C*H*$_3$CHH); 100-MHz ^{13}C NMR ($CDCl_3$) δ 151.2 (enriched, d, J_{CC} = 70.63 Hz), 121.4 (enriched, d, J_{CC} = 70.73 Hz), 75.8 (d, J_{CC} = 3.2 Hz), 60.2 (d, J_{CC} = 1.3 Hz), 42.1 (d, J_{CC} = 41.7 Hz), 27.3, 14.2, 13.9, 10.2; $[\alpha]_D$ = 35.16° ($CHCl_3$, l = 1.0 dm, 0.0442 g/mL); CIMS (NH_4^+) (M + H)$^+$ 189 m/e.

(4*R*,5*R*)-4-Methyl-5-hydroxy-2-heptenoic Acid (14). Unlabeled ester 13 (418 mg, 2.25 mmol), 22 mL, 0.5 M K_2CO_3 (11.24 mmol, 5.0 equiv), and 110 mL of 5:3 THF/H_2O (69 mL THF/41 mL H_2O) were refluxed together for 24 h. After removal of THF, the basic aqueous mixture was extracted with 2 × 50 mL of CH_2Cl_2 and acidified to pH 3.0 with 5% HCl, and the acid was extracted with 3 × 50 mL of CH_2Cl_2 to yield 255 mg of pure acid in 71.7% yield: IR ($CHCl_3$) ν 3407, 3019, 2971, 2937, 2879, 2678, 1698, 1654, 1282 cm^{-1}; 250-MHz ^1H NMR ($CDCl_3$) δ 7.07 (dd, J = 7.8, 16.3 Hz, 1 H, C*H*=CHCO$_2$), 5.87 (dd, J = 1.12, 15.7 Hz, 1 H, CH=C*H*CO$_2$), 3.53 (m, 1 H, C*H*OH), 2.48 (m, 1 H, C*H*CH$_3$), 1.64–1.25 (m, 2 H, CH$_3$C*H*$_2$), 1.10 (d, J = 6.8 Hz, CHC*H*$_3$), 0.92 (t, J = 7.4 Hz, 3 H, C*H*$_3$CH$_2$); 100-MHz ^{13}C NMR ($CDCl_3$) δ 171.3, 153.8, 120.8, 75.9, 42.2, 27.3, 13.7, 10.2; $[\alpha]_D$ = +43.33° ($CHCl_3$, l = 1.0 dm, 0.018 g/mL); CIMS (NH_4^+) (M + H)$^+$ 159 m/e.

(4*R*,5*R*)-[2,3-$^{13}C_2$]-4-Methyl-5-hydroxy-2-heptenoic Acid (14). (4*R*,5*R*)-[1,2-$^{13}C_2$]-4-Methyl-5-hydroxy-2-heptenoic acid ([2,3-$^{13}C_2$]-14) was prepared from ethyl (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoate following the same procedure described for unlabeled 14: 400-MHz ^1H NMR ($CDCl_3$) δ 7.06 (dddd, J = 1.93, 7.78, 15.74, 154.5 Hz, 1 H, *H*^{13}C=^{13}CHCO$_2$), 6.7 (b, 1 H, CO$_2$H), 5.86 (dd, J = 15.6, 162.7 Hz, 1 H, H^{13}C=^{13}C*H*CO$_2$), 3.54 (m, 1 H, C*H*OH), 2.47 (m, 1 H, C*H*CH$_3$), 1.6–1.5 (m, 1 H, CH$_3$C*H*H), 1.45–1.35 (m, 1 H, CH$_3$CH*H*), 1.10 (dd, J = 4.86, 6.78 Hz, 3 H, CHC*H*$_3$), 0.97 (t, J = 7.40 Hz, 3 H, C*H*$_3$CHH); 100-MHz ^{13}C NMR ($CDCl_3$) δ 153.7 (enriched, d, J_{CC} = 70.0 Hz), 120.8 (enriched, d, J_{CC} = 70.1 Hz).

Thallous *N*-Acetylcysteamine.[16] Thallous ethoxide (325 μL, 1.18 g, 459 mmol) was added via syringe to an oven-dried 100-mL round-bottom flask under argon atmosphere containing 20 mL of dry THF. *N*-Acetylcysteamine (574 mg in 20 mL of dry THF) was added dropwise via cannula over 30 min at room temperature. Upon addition of the *N*-acetylcysteamine the solution turned a bright yellow to yield a suspension with a final concentration of 0.12 M.

***N*-Acetylcysteamine Thioester of (4*R*,5*R*)-4-Methyl-5-hydroxy-2-heptenoic Acid (10a).**[16,17] In an oven-dried 10-mL RB flask, 57 mg of unlabeled 14 (0.36 mmol) and 50 μL of Et_3N (0.36 mmol, 1.0 equiv) in 3 mL of dry THF was allowed to stir under an argon atmosphere at room temperature for 10 min. To this mixture was added 52 μL of diethylchlorophosphonate (0.36 mmol, 1.0 equiv) in 1 mL of dry THF dropwise. The reaction was stirred for 3 h after which the fine white Et_3N·HCl salt was filtered out via Schlenk filtration and rinsed with 2 × 10 mL of THF. The solvent was removed and the phosphono anhydride concentrated to an oil. This oil was then dissolved in 12 mL of dry THF in a 50-mL RB flask equipped with a stir bar and septum. The solution was cooled to 0 °C, and 3 mL of thallous *N*-acetylcysteamine as a bright yellow suspension in THF (0.12 M) was added dropwise via cannula. The reaction was allowed to stir at 0 °C for 4 h, after which time the yellow color had dissipated to yield a white suspension. The solvent was removed by rotary evaporation, and the crude was taken up in Et_2O, filtered through Celite, and concentrated to 123 mg of oil. Flash silica gel chromatography (4% MeOH/$CHCl_3$) provided 53 mg of oil (57%): R_f = 0.35 (10% MeOH/0.1% NH_4OH/$CHCl_3$); IR (neat) ν 3303, 3086, 2970, 1660, 1551, 1290, 979 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 6.95 (dd, J = 7.72, 15.64 Hz, 1 H, C*H*—CHCO$_2$), 6.25 (b, 1 H, N*H*), 6.16 (dd, J = 1.26, 15.66 Hz, 1 H, CH—C*H*CO$_2$), 3.52 (m, 1 H, C*H*OH), 3.45 (m, 2 H, C*H*$_2$NHAc), 3.09 (t, J = 6.4 Hz, 2 H, SC*H*$_2$), 2.45 (m, 1 H, C*H*CH$_3$), 2.22 (b, 1 H, CHO*H*), 1.97 (s, 3 H, COC*H*$_3$), 1.57–1.51 (m, 1 H, CH$_3$C*H*H), 1.44–1.39 (m, 1 H, CH$_3$CH*H*), 1.10 (d, J = 6.83 Hz, 3 H, CHC*H*$_3$), 0.97 (t, J = 7.39 Hz, 3 H, C*H*$_3$CH$_2$); 100-MHz ^{13}C NMR ($CDCl_3$) δ 190.3, 170.4, 148.5, 128.1, 75.7, 42.1, 39.6, 28.3, 27.3, 23.1, 13.7, 10.2; $[\alpha]_D$ = +31.92° ($CHCl_3$, l = 1.0 dm, 0.0026 g/mL); CIMS (NH_4^+) (M + H)$^+$ 260 m/e; exact mass calcd for $C_{12}H_{22}NO_3S$ 260.1320, found 260.1338.

***N*-Acetylcysteamine Thioester of (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-Methyl-5-hydroxy-2-heptenoic Acid (10a).** *N*-Acetylcysteamine thioester of (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptonoic acid ([2,3-$^{13}C_2$]-8) was prepared from (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoic acid following the same procedure as described for unlabeled 10a: IR (neat) ν 3311, 3085, 2970, 2878, 1658, 1574, 1288, 1218, 758 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 6.91 (dddd, J = 1.49, 7.70, 15.6, 153.7 Hz, 1 H, *H*^{13}C=^{13}CHCO$_2$), 6.4 (b, 1 H, N*H*), 6.14 (dd, J = 15.6, 161.5 Hz, 1 H H^{13}C=^{13}C*H*CO$_2$), 3.50 (m, 1 H, C*H*OH), 3.38 (dt, J = 6.20, 6.30 Hz, 2 H, C*H*$_2$NHAc), 3.05 (t, J = 6.56 Hz, 2 H, SC*H*$_2$), 2.55 (b, 1 H, O*H*), 2.40 (m, 1 H, C*H*CH$_3$), 1.55–1.45 (m, 1 H, CH$_3$C*H*H), 1.41–1.30 (m, 1 H, CH$_3$CH*H*), 1.06 (dd, J = 4.9, 6.8 Hz, 3 H, CHC*H*$_3$), 0.93 (t, J = 7.39 Hz, 3 H, C*H*$_3$CHH); 100-MHz ^{13}C NMR ($CDCl_3$) δ 149.0 (enriched, d, J_{CC} = 69.9 Hz), 128.3 (enriched, d, J_{CC} = 69.9 Hz); $[\alpha]_D$ = 27.6° ($CHCl_3$, l = 1.0 dm, 0.0389 g/mL); CIMS (NH_4^+) (M + H)$^+$ 262 m/e.

Acknowledgment. This work was supported by a grant from the National Institutes of Health, GM22172, and by the NIH-(RR02231)–USDOE/OHER Stable Isotope Resource program at Los Alamos. We also thank Dr. William L. Parker of the Squibb Institute for Medical Research, Princeton, NJ, for providing strains of *S. venezuelae* SC2366 and an authentic sample of methymycin.